Docket: NEB-177-PUS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: Evans et al. EXAMINER: Schnizer

SERIAL NO.: 09/937,070 GROUP: 1653

FILED: January 29, 2002

FOR: Method for Producing Circular or Multimeric Protein Species *in vivo* or *in vitro* and Related Methods

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Certificate of Transmission

I hereby certify that this correspondence to the Commissioner for Patents, Attn: Examiner Holly Schnizer is being transmitted via facsimile to ~~(703) 872-9306~~ (571)273-8300

Leslie Goldberg

Sir:

DECLARATION UNDER 37 C.F.R. §1.131

As a below named inventor, I hereby declare that:

1. My name is Dr. Ming-Qun Xu, Senior Scientist at New England Biolabs Inc. My resume is attached. While Tom Evans and I worked together on the claimed invention, the attached notebook pages are obtained from my laboratory notebook.

2. The Examiner has rejected claims 12, 14 and 16 in the above application, directed to a method for the *in vivo* production of a cyclic polypeptide, citing Scott et al. *PNAS* 96, pp 13638-13643 which was submitted October 7, 1999 and published November 23, 1999. In support of our assertion that we conceived and diligently reduced to practice the claimed invention prior to November 23, 1999 and indeed prior to October 7, 1999, copies of three pages from the laboratory notebook of Ming-Qun Xu are attached with redaction of the dates. A discussion of each page is presented below. Applicants submitted a paper for publication on December 8, 1999 that was published March 31, 2000 (copy attached hereto). References to this paper are made in conjunction with the discussion of the earlier completed experimental data attached hereto.

3. On the first page of the lab notebook attachment, a sequence for maltose-binding protein (MBP) is shown that was determined after Factor Xa cleavage. This sequence established that the MBP had been circularized by *in vivo* splicing. The sequence obtained corresponded to the C-terminal end of the protein (GTLEKFAEY) adjacent to the N-terminus (C here X, FNISTGM). The diagram on the left of the sequence depicts a circular molecule with the C-terminal end of MBP adjacent to the N-terminus. The third diagram on the page shows the N-terminal end of the MBP protein (XFNISTGM) that results when a single split intein is cleaved at the N-terminal end of the MBP protein. The first diagram on the page provides the sequence of the split intein (referring to Ssp DnaE intein) in the intein-MBP fusion protein, identified as MEB 16, in the absence of cleavage.

4. The second submitted page shows the sequence associated with two bands from a gel where the higher band is suspected of being circular MBP while the lower band is thought to be linear MBP following intein cleavage. Amino acid sequencing confirmed these assumptions. This data is also provided on page 9094 of the attached Evans reference, with the paragraph identified by (1).

5. Page 181 of the lab notebook shows a cartoon at the top of the page describing the design of the experiment to test whether splicing of the split intein occurred and if so whether linear or circular MBP was produced from splicing of both split inteins from the ends of the MBP protein. The gel also present on the page shows that in fact circularized MBP is obtained as seen by a particular band on the gel (lane 6) and that the circular protein band disappears after cleavage with Factor Xa to produce a band corresponding to linear MBP (lane 7). This gel is reproduced in Figure 5A of the Evans reference (p. 9093) after the experiment was repeated. Lane 4 in Figure 5A corresponds to lane 6 on the gel on attached lab notebook page 181 (read from left to right) and lane 5 in Figure 5A corresponds to lane 7 of the gel of page 181.

6. The evidence provided above teaches for the first time the *in vivo* production of a cyclic polypeptide.

7. I further declare under penalty of perjury pursuant to laws of the United States of America the foregoing is true and correct.

Dr. Ming Xu

2/24/06
Date

Ming-Qun Xu, Ph.D.

Senior Scientist

New England Biolabs, Inc.

240 County Road

Ipswich, MA 01938-2723 USA

Phone: (978) 380-7241

Fax: (978) 921-1350

E-mail: xum@neb.com

Education:

B.S. 1982 University of Science and Technology of China

Ph.D. 1989 Molecular Biology, Department of Biological Sciences, State University of New York at Albany

Work History

1990-1992: Postdoctoral research on self-splicing introns with Dr. David Shub, SUNY at Albany
Discovered the first eubacterial intron (Xu et al. Science, 1990)

1992 – 1994: Postdoctoral research with Dr. Fran Perler at New England Biolabs, Inc. Performed the first in vitro protein splicing experiment (Xu et al., Cell 1993)

1994 – 1997: Staff Scientist at New England Biolabs, Inc. Investigated the chemical mechanism of protein self-splicing. Developed the intein-based affinity purification system – IMPACT.

1997 – present: Senior Scientist at New England Biolabs, Inc. Structural and mechanistic studies of self-splicing inteins. Engineered inteins for protein semisynthesis, protein backbone cyclization and trans-splicing.

2001-2005: Managing Director, New England Biolabs (Beijing) Ltd.

Current Research Interest:

Structural and mechanistic studies of protein splicing have been conducted by collaboration to solve the crystal structures of self-splicing-inteins derived from the *dna*B and *dna*E genes of *Synechocystis* sp. PCC6803. The finding that the DnaE intein precursor structure contains a zinc ion, the only identified inhibitor of both *cis*- and *trans*-splicing, chelating the highly conserved Cys160 and Asp140 reveals the structural basis of Zn^{2+}-mediated inhibition. These structural

studies provide insight into the sequential reaction property of protein splicing as well as the strategies to utilize inteins for protein engineering.

A number of intein engineering projects have been carried out for protein/antibody affinity purification, protein labeling and tagging, ligation and cyclization of expressed proteins. The Intein-mediated protein ligation (IPL) method has been applied to new fields including antibody characterization, epitope mapping, kinase/phosphatase assays for analysis via peptide arrays, western blots and ELISA.

PUBLICATIONS:

1. Ming-Qun Xu, Inca Ghosh, Samvel Kochinyan and Luo Sun. Intein-mediated Peptide Arrays for Epitope Mapping and Kinase/Phosphatase Assays. *Methods in Molecular Biology, vol., Microarrays: Methods and Protocols Edited by J.B. Rampal. Humana Press Inc., Totowa, NY. In press.*
2. Sun Ping, Sheng Ye, Sebastien Ferrandon, Evans, T.C. Jr., Ming-Qun Xu, Zihe Rao (2005) Crystal structures of an intein from the split *dnaE* gene of *Synechocystis* sp. PCC6803 reveal the catalytic model of intein without the penultimate histidine and the mechanism of zinc ion inhibition of protein splicing. *J. Mol. Biol. 353: 1093-1105.*
3. Xu, M.-Q. and Evans, T.C. Jr. (2005) Recent Advances in Protein Splicing: Manipulating Proteins In Vitro and In Vivo. *Curr. Opin. Biotechnol.* 16, 440-446.
4. Guo CY, Li ZY, Xu MQ, Yuan JM. (2005) Preparation of an immunoadsorbent coupled with a recombinant antigen to remove anti-acetylcholine receptor antibodies in abnormal serum. J Immunol Methods. 2005 Aug;303(1-2):142-7.
5. Shaorong Chong and Ming-Qun Xu. (2005) Harnessing Inteins for Protein Purification and Characterization. Marlene Belfort (Ed.) *Nucleic Acids and Molecular Biology*, Vol. 16, 273-292.
6. Evans, T.C. Jr., Xu, M.-Q. and Pradhan S. (2005) Protein Splicing Elements and Plants: From Transgene Containment to Protein Purification. *Annu. Rev. Plant Biol.* (2005) 56:375-92..
7. Sun, L., Rush, J., Ghosh, I., Maunus, J.R. and Xu, M.-Q. (2004) Producing peptide arrays for epitope mapping by intein-mediated protein ligation. *Biotechniques*. 37: 430-443.
8. Ghosh, I, Sun, L., Evans, T.C. Jr., and Xu, M.-Q. (2004) An improved method for utilization of peptide substrates for antibody characterization and enzymatic assays. J. of Imm. Methods. 293:85-95
9. Xu, J., Sun, L., Ghosh, I., and Xu, M.-Q. (2004) Western blot analysis of Src kinase assays using peptide substrates ligated to a carrier protein. *Biotechniques*. 36:976 -981.
10. Li ZY, Li YJ, Guo CY, Shi YW, Xu MQ, Trommer WE, Yuan JM. (2004) Soluble expression and affinity purification of functional domain of human acetylcholine receptor alpha-subunit by the modulation of maltose binding protein. Biotechnol Lett. 2004 Dec;26(23):1765-9.

11. Guo C, Li Z, Shi Y, Xu M, Wise JG, Trommer WE, Yuan J. (2004) Intein-mediated fusion expression, high efficient refolding, and one-step purification of gelonin toxin. Protein Expr Purif. 2004 Oct;37(2):361-7.

12. Luo Sun, Inca Ghosh, Ming-Qun Xu. (2003) Generation of an affinity column for antibody purification by intein-mediated protein ligation. Journal of Immunological Methods 282, 45-52.

13. Yi Ding, Ming-Qun Xu, Inca Ghosh, Xuehui Chen, Sebastien Ferrandon, Guillaume Lesage, Zihe Rao (2003) Crystal structure of a mini-intein reveals a conserved catalytic module involved in side chain cyclization of asparagine during protein splicing. *J. Biol. Chem.* 278, 39133-39142.

14. Hang Gyeong Chin et al. (2003) Protein trans-splicing in transgenic plant chloroplast: Reconstitution of herbicide resistance from split genes. *PNAS 100,* 4510-4515.

15. Ferrandon, S., Sterzenbach, T., Mersha, F. and M.-Q. Xu. (2003) A single surface tryptophan in the chitin-binding domain from *Bacillus circulans* chitinase A1 plays a pivotal role in binding chitin. *Biochim. Biophys. Acta. 1621,* 31-40.

16. Evans, T.C. Jr. and M.-Q. Xu. (2002) Purification of recombinant proteins from *E. coli* by engineered inteins. *Methods in Molecular Biology, vol. 205, E. coli Gene Expression Protocol Edited by P.E. Vaillancourt. Humana Press Inc., Totowa, NY*

17. Xu, M.-Q., Chong, S., and Evans, T. C. Jr. (2001) Protein splicing. *Encyclopedia of Genetics.*

18. Xu, M.-Q., and Evans, T. C. Jr. (2001) Intein-mediated ligation and cyclization of expressed proteins. *Methods* 24, 257-277.

19. Ghosh, I., Sun, L. and Xu, M.-Q. (2001) Zinc inhibition of protein *trans*-splicing and identification of regions essential for splicing and association of a split intein. *J. Biol. Chem.* 276, 24051-24058.

20. Martin, D.D., Xu, M.-Q., Evans, T. C. (2001) Characterization of a naturally occurring trans-splicing Intein from *Synechocystis sp.* PCC6803. *Biochemistry* 40:1393-1402.

21. Sun, L., Ghosh, I., Paulus, H., and Xu, M.-Q. (2001) Protein *trans*-splicing to produce herbicide-resistant acetolactate synthase. *Applied Environ. Microbio.* 67, 1025-1029.

22. Xu, M.Q., Paulus, H. and Chong, S. (2000) Fusions to self-splicing inteins for protein purification. *Methods Enzymol.* 326, 376-418.

23. Poland, B. W., Xu, M.Q., Quiocho, F.L (2000). Structural Insights into the Protein Splicing Mechanism of PI-Scel. *J. Biol. Chem. 275, 16408-16413.*

24. Yuan, J.M., Li, Z.Y., Wang, Y.M., and Xu, M.-Q. (2000) One step purification of recombinant human Neurotrophic factor-3 with the splicing function of intein. *Chin. J. Biochem. Mol. Biol.* 16, 335-339.

25. Evans, T.C. Jr., Martin, D., Kolly R., Panne, D. , Sun, L., Ghosh, I., Chen, L., Benner, J., Liu, X.-Q., and Xu, M.-Q. (2000) Protein *trans*-splicing and cyclization by a naturally split intein from the *dnaE* gene of *Synechocystis* species PCC6803. *J. Biol. Chem.* 275, 9091-9094.

26. Evans, T.C. Jr., and and Xu, M.-Q. (1999). Intein-mediated Protein Ligation: Harnessing Nature's Escape Artists. *Biopolymers* 51, 333-342.

27. Evans, T.C. Jr., Benner, J., and Xu, M.-Q. (1999) The cyclization and polymerization of bacterially-expressed proteins using modified self-splicing inteins. *J. Biol. Chem.* 274, 18359-18363.

28. Evans, T.C. Jr., Benner, J., and Xu, M.-Q. (1999). The *in vitro* ligation of bacterially expressed proteins using an intein from *Methanobacterium thermoautotrophicum. J. Biol. Chem.* 274, 3923-3926.

29. Mathys, S., Evans, T.C. Jr., Chute, I.C., Wu, H., Chong, S., Benner, J., Liu, X.-Q., Xu, M.-Q. (1999) Characterization of a self-splicing mini-intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation. *Gene* 231:1-13.

30. Shi, Y., Fan, J., Li, Z. Yuan, J., Xu, M.-Q., Chong S. (1999). A new protein splicing system and its splicing conditions. *Chin. J. Biochem. Mol. Biol.* 15, 88-91.

31. Southworth, M.W., Amaya, K., Evans, T.C., Xu, M.-Q., and Perler, F.B. (1999) Purification of proteins fused to either the amino or carboxy terminus of the Mycobacterium xenopi Gyrase A intein. *BioTechniques* 27, 110-120.

32. Evans, T.C. Jr., Benner, J., Xu, M.-Q., 1998. Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci.* 7, 2256-2264.

33. Chong, S., Montello, G.E., Zhang, A., Cantor, E.J., Liao, W., Xu, M.-Q., Benner, J., 1998. Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step. *Nucleic Acids Res.* 26, 5109-5115.

34. Chong, S., Williams, K.S., Wotkowicz, C.and Xu, M.-Q. 1998. Modulation of protein splicing of the *Saccharomyces cerevisiae* vacuolar membrane ATPase intein. *J. Biol. Chem* 273, 10567-10577.

35. Wu, H., Xu, M.-Q., Liu, X.-Q., 1998. Protein trans-splicing and functional mini-inteins of a cyanobacterial DnaB intein. *Biochim. Biophys. Acta* 1387, 422-432.

36. Perler, F., Xu, M.-Q., and Paulus, H. 1997. Protein splicing and autoproteolysis mechanisms. *Curr. Opin. in Biotechnol.* 1, 292-299.

37. Chong, S., Mersha, F.B., Comb, D.G., Scott, M. E., Landry, D., Vence, L.M., Perler, F.B., Benner, J., Kucera, R.B., Hirvonen, C.A., Pelletier, J.J., Paulus, H., and Xu, M.-Q. (1997) Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* 192, 271-281.

38. Chong, S. and Xu, M.-Q. (1997) Protein splicing of the *Saccharomyces cerevisiae* VMA intein without the endonuclease motifs. *J. Biol. Chem.* 272, 15587-15590.

39. Chong, S., Shao Y., Paulus, H, Benner, J., Perler, F.B., and Xu, M.-Q. (1996). Protein splicing involving the *Saccharomyces cerevisiae* VMA intein: the steps in the splicing pathway, side reactions leading to protein cleavage, and establishment of an *in vitro* splicing system. *J. Biol. Chem.* 271, 22159-22168.

40. Xu, M.-Q. and Perler, F. B. (1996). The mechanism of protein splicing and its modulation by mutation. *EMBO J.* 15, 5146-5153.

41. Shao, Y., Xu, M.-Q., and Paulus, H. (1996). Protein splicing: Evidence for an N-O acyl rearrangement as the initial step in the splicing process. *Biochemistry.* 35, 3810-3815.

42. Shao, Y., Xu, M.-Q., and Paulus, H. (1995). Protein splicing: characterization of the aminosuccinimide residue at the carboxyl terminus of the excised intervening sequence. *Biochemistry.* 34, 10844-10850.

43. Xu, M.-Q., Comb, D. G., Paulus, H., Noren, C. J., Shao, Y., and Perler, F. B. (1994). Protein splicing: an analysis of the branched intermediate and its resolution by succinimide formation. *EMBO J.* 13, 5517-5522.

44. Xu, M.-Q., Southworth, M. W., Mersha, F. B., Hornstra, L. J., and Perler, F. B. (1993) *In vitro* protein splicing of purified precursor and the identification of a branched intermediate. *Cell* 75, 1371-1377.

45. Michel, F., L. Jaeger, E. Westhof, R. Kuras, F. Tihy, M.-Q. Xu, and D.A. Shub.(1992). Activation of the catalytic core of a group I intron by a remote 3' splice junction. *Genes and Dev.* 6, 1373-1385.

46. Xu, M.-Q., S.D. Kathe, H. Goodrich-Blair, S.A. Nierzwicki-Bauer, and D.A. Shub. (1990). Bacterial origin of a chloroplast intron: conserved self-splicing group I introns in cyanobacteria. *Science* 250, 1566-1570.

47. Michel, F., P. Netter, M.-Q. Xu and D.A. Shub. 1990. Mechanism of 3' splice site selection by the catalytic core of the *sunY* intron of bacteriophage T4: the role of a novel base pairing interaction in group I introns. *Genes & Dev.* 4, 777-788.

48. Goodrich, H.A., Vincenzo Scarlato, J.M. Gott, M.-Q. Xu, and D.A. Shub. 1990. A group I intron in Bacillus subtilis bacteriophage SPO1. *Cell* 63, 417.

49. Goodrich, H.A., J.M. Gott, M.-Q. Xu, Vincenzo Scarlato, and D.A. Shub. (1989). A group I intron in *Bacillus subtilis* bacteriophage SPO1. *Molecular Biology of RNA*. Alan R. Liss, Inc. 59-66.

50. Xu, M.-Q. and D.A. Shub. (1989). The catalytic core of the *sunY* intron of bacteriophage T4. *Gene* 82, 77-82.

51. Shub, D.A., J.M. Gott, M.-Q. Xu, B.F. Lang, F. Michel, J. Tomaschewski, J. Pedersen-Lane, and M. Belfort. (1988). Structural conservation among three homologous introns of bacteriophage T4 and the group I introns of eukaryotes. *Proc. Natl. Acad. Sci.* 85, 1151-1155.

52. Shub, D.A., M.-Q. Xu, J.M. Gott, A. Zeeh, and L.D. Wilson. 1987. A family of autocatalytic group I introns in bacteriophage T4. *Cold Spring Harbor Symp. Quant. Biol.* 52, 193-200.